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Factors Involved in the Cytotoxicity of UICC Amosite towards Macrophages

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The cytotoxicity of various ball-milled UICC amosite samples toward mouse peritoneal macrophages *in vitro* was examined. On increased ball milling, the cytotoxicity of the amosite samples fell. This decreased biological activity seemed to correlate best with number of fibers longer than 4 μm/μg dust, rather than total number of fibers or the electrophoretic mobility of the amosite samples.

It has been shown that the carcinogenicity of fibrous minerals depends on morphological criteria and not on the chemistry of the materials (*1*). The appearance of pleural mesotheliomas (following direct implantation in the pleural cavity) correlated best with increasing numbers of fibers having both diameters of 0.25 μm or less and lengths of more than 8 μm, but relatively high correlations were also noted with fibers having diameters of up to 1.5 μm and lengths greater than 4 μm. Some *in vitro* studies (*2, 3*) have demonstrated that fiber dimensions also play a role in the cytotoxic effect of various minerals towards mammalian cells. One of the easiest ways to produce experimental samples for such studies is by ceramic ball milling, the principal effect of which (on amphibole asbestos) is to reduce the length of the fibers (*2*). However, Langer et al. (*4*) and Spurny et al. (*5*) have demonstrated that ball milling may produce other changes in the physical and chemical properties of the original fiber samples; e.g., such manipulation decreases the fiber's crystallinity. It is already recognized that the grinding of quartz samples produces a "disturbed" layer on the particles (*6*), removal of which by etching enhances the pathogenic effects of the dusts toward animals (*7*) and macrophages (*8*).

The role of surface charge on minerals in the biological effects of dusts has been little investigated due to the time-consuming nature of the determination of electrophoretic mobility using conventional microelectrophoresis apparatus. Light and Wei (*9*) showed that the zeta potential of UICC chrysotile A and crocidolite changed on leaching with HCl or Tyrode's solution and there was a significant correlation between zeta potential and the haemolytic activity of chrysotile and crocidolite.

In this paper we used various ball-milled UICC amosite samples in order to determine the parameters that were important in the dusts, cytotoxicity towards mouse peritoneal macrophages. The surface charge of the samples was determined by laser Doppler velocimetry, a new and rapid method for determining the electrophoretic mobility of a wide range of materials (*10*). The size distribution of the fibers in the samples was determined by transmission electron microscopy.

Materials and Methods

Culture of Macrophages with Dusts

Unstimulated mouse peritoneal macrophages were obtained by lavage of 22-27 g female T.O. mice (Tuck and Son Ltd, Battlesbridge, Essex) with 3 cm^3 Medium 199 (Flow Laboratories, Irvine, Scotland) containing 5 IU heparin, 100 U benzylpenicillin and 100 μg streptomycin/cm^3. Approximately 1.2×10^6 cells (in 2 mL of the above medium) were added to each well of Linbro tissue culture multi-well plates (Flow Laboratories, Irvine, Scotland; well diameter 24 mm), and left for 1 hr at 37°C in a 5% CO_2/95% air atmosphere.

At the end of the period, the nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and 2 cm^3 Medium 199 containing the

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above antibiotics, and 10% newborn calf serum (Flow Laboratories, Irvine, Scotland) was added. This serum had previously been heat-inactivated (56°C for 30 min) and acid-treated (*11*). Cultures were then left for 24 hr at 37°C in a 5% CO_2/95% air atmosphere before addition of fresh medium containing dusts.

Stock amosite solutions were made up in PBS at a concentration of 1.5 mg/mL, ultrasonication being used to disperse the dust. The amosite was added to the above medium containing 10% serum at a concentration of 60 $\mu g/cm^3$ and left for 2-3 hr at 37°C before addition to the macrophage cultures. A 2 mL portion of "dusted" medium was added to each four culture dishes which were then incubated for another 18 hr.

At the end of this period the medium was collected, and the adherent cells disrupted by addition of 2 mL saline containing 0.1% Triton X-100 and 0.1% bovine serum albumin, and by rubbing the dish with a sterile siliconized rubber bung. Both medium and cell lysates were centrifuged at 500*g* for 10 min and the supernatants assayed for lactate dehydrogenase (LDH) by the continuous flow fluorimetric method of Morgan et al. (*12*) by use of a Perkin-Elmer Model 3000 fluorescence spectrometer.

The release of LDH from the cells into the culture medium is an indicator of dust cytotoxicity and is calculated as:

$$\%\ \text{enzyme released into culture medium} = [M/(M + C)] \times 100$$

where M is the enzyme activity of the medium and C is the enzyme activity of the cell lysate. The mean percent enzyme released for the four cultures used for each dust treatment, together with the 95% confidence intervals, was calculated.

Particle Size Analysis

Samples of UICC amosite (*13*) were ball-milled for various times in a ceramic ball mill according to the method of Brown et al. (*2*). A distilled water suspension (10-40 $\mu g/cm^3$) of each amosite sample was drawn through a 25 mm 0.1 μm pore size filter (Millipore) in a Sartorius membrane filter holder. The filter was removed and coated with carbon in a Nanotech coating plant. Electron microscope grids (Graticule HF32 100 mesh) were placed on a 0.5 cm thick plastic sponge soaked in acetone, and the carbon-coated filters transferred to the top of the grids. After 15 min, sufficient of the filter material had dissolved for the grids to be removed and examined in the electron microscope. Electron micrographs of overlapping fields were taken at $\times 1500$ magnification; the prints were then assembled into mosaics. This method allows the measurement of very long and short fibers on the same micrographs. The length of each fiber was measured with a millimeter rule and its diameter with a Trimbrell-Coulter Shearicon; about 300 fibers were measured for each size distribution. The numbers of fibers in unit mass of each dust were calculated from the area of micrograph examined and the mass of dust deposited on each filter.

Laser Doppler Velocimetry (LDV)

Details of the method used are as described by Preece and Luckman (*14*). In principle, the method is used to measure the Doppler shift of the frequency of the scattered light due to the velocity of the scattering particles in an electric field. In practice, the laser Doppler cytopherometer has crossed beams producing interference fringes in the center of a glass capillary. Dust particles suspended in an appropriate solution are introduced and an electrical field applied. The movement of the dust particles interacts with the fringes, producing a "differential Doppler" signal which is collected by suitable optics, detected by a photomultiplier and processed by a spectrum analyzer to produce a power spectrum of the photocurrent.

It is a design difficulty that it is not easy to identify and locate a stationary layer in small capillary (0.7 mm); thus, in all cases a coated capillary was used (*15*). The presence of the methylcellulose inhibits electro-osmosis by locating the effective plane of shear within a high viscosity matrix. The reduction in electro-osmosis allowed the center zone to be used. Performance of the coating was checked before and after each series by using a suspension of fresh erythrocytes in physiologically compatible medium, or, a stock of glutaraldehyde-fixed erythrocytes in low ionic strength media.

The lifetime of capillaries varied considerably with the material under study, e.g., kaolinite caused rapid destruction of the coating necessitating frequent replacement. The electrophoretic mobility of the dust specimens was compared with that of human erythrocytes, the mobility of which is 1.07 ± 0.02 μm/sec-V/cm at 25°C in phosphate-buffered saline (Dulbecco's diluted threefold in water).

The polarity of charge was observed by viewing perpendicularly to the laser beams and noting the direction of flow of the points of light from the scattering particles.

Results and Discussion

The cytotoxicity of the ball-milled UICC amosite samples (as measured by the release of lactate dehydrogenase from cells) towards macrophages is

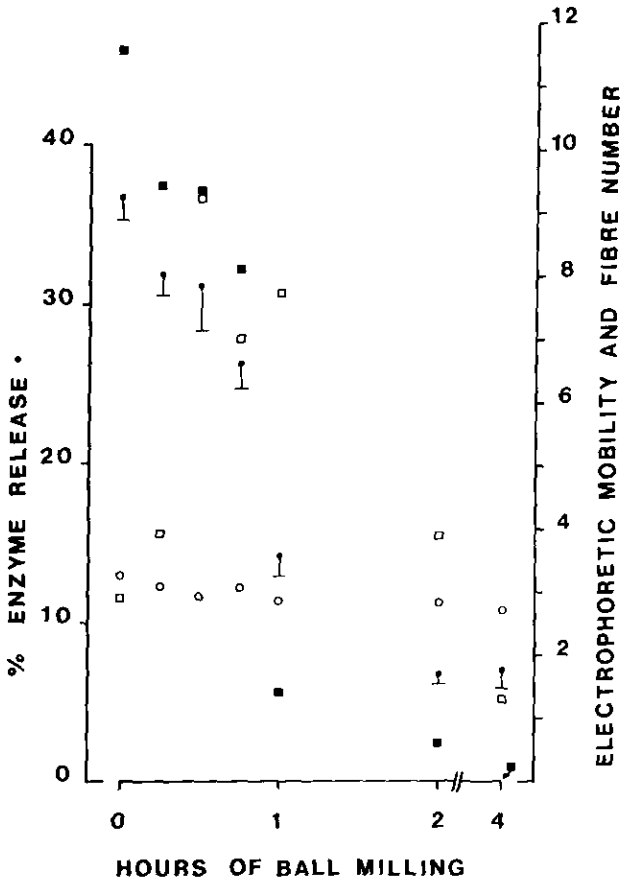

FIGURE 1. Release of lactate dehydrogenase from ball-milled UICC amosite treated macrophages: (●) % lactate dehydrogenase ± 95% confidence limits; (○) electrophoretic mobility of amosite samples (μm/sec-V/cm); (□) total number of fibers in amosite samples × 10^5; (■) total number of fibers in amosite samples ⩾ 4 μm (× 10^4).

shown in Figure 1. As the ball milling time increases, the cytotoxicity of the samples falls dramatically.

In Figure 1 we also show the electrophoretic mobility (EM) of the various amosite samples. Increased ball milling has had a small effect on the mobility of the particles; after 4 hr ball milling the EM is 2.70 μm/sec-V/cm compared with 3.25 for the parent UICC amosite. It seems unlikely that this change in EM was the main factor involved in the large fall in the cytotoxicity of milled UICC amosite towards macrophages. These results do not exclude the possibility that the surface charge on amosite plays some role in its biological effect; however, it strongly suggests that an additional factor is playing an important role.

Figure 1 shows that on ball milling there is a significant rise in the number of fibers (defined as particles with a length/diameter ratio ⩾ 3)/μg dust, reaching a peak after 0.5 hr and declining thereafter. This increase in fiber number arises from the fracturing of the longer fibers during the early period of milling. Clearly the cytotoxicity of the amosite does not depend on total fiber number as this value is rising while the cytotoxicity is falling.

Figure 1 also shows the number of fibers having lengths of ⩾ 4 μm per microgram of dust. With increased ball milling time there is a fall in the number of such fibers. This mirrors the fall in the cytotoxicity of the dusts towards macrophages.

Marks and Nagelschmidt (*8*) and Styles and Wilson (*16*) have indicated that there is a degree of correlation between the cytotoxicity of dusts toward macrophages *in vitro* and the ability of dusts to cause fibrosis in animal studies. The low cytotoxicities of the amosite ball-milled 2 or 4 hr would suggest that these materials would have a low fibrogenic potential, but there are no published data to support this prediction.

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